Selective Peroxidation and Externalization of Phosphatidylserine in Normal Human Epidermal Keratinocytes During Oxidative Stress Induced by Cumene Hydroperoxide

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Reactive oxygen species not only modulate important signal transduction pathways, but also induce DNA damage and cytotoxicity in keratinocytes. Hydrogen peroxide and organic peroxides are particularly important as these chemicals are widely used in dermally applied cosmetics and pharmaceuticals, and also represent endogenous metabolic intermediates. Lipid peroxidation is of fundamental interest in the cellular response to peroxides, as lipids are extremely sensitive to oxidation and lipid-based signaling systems have been implicated in a number of cellular processes, including apoptosis. Oxidation of specific phospholipid classes was measured in normal human epidermal keratinocytes exposed to cumene hydroperoxide after metabolic incorporation of the fluorescent oxidation-sensitive fatty acid, *cis*-parinaric acid, using a fluorescence high-performance liquid chromatography assay. In addition, lipid oxidation was correlated with changes in membrane phospholipid asymmetry and other markers of apoptosis. Although cumene hydroperoxide produced significant oxidation of *cis*-parinaric acid in all phospholipid classes, one phospholipid, phosphatidylserine, appeared to be preferentially oxidized above all other species. Using fluorescamine derivatization and annexin V binding it was observed that specific oxidation of phosphatidylserine was accompanied by phosphatidylserine translocation from the inner to the outer plasma membrane surface where it may serve as a recognition signal for interaction with phagocytic macrophages. These effects occurred much earlier than any detectable changes in other apoptotic markers such as caspase-3 activation, DNA fragmentation, or changes in nuclear morphology. Thus, normal human epidermal keratinocytes undergo profound lipid oxidation with preference for phosphatidylserine followed by phosphatidylserine externalization upon exposure to cumene hydroperoxide. It is therefore likely that normal human epidermal keratinocytes exposed to similar oxidative stress *in vivo* would undergo phosphatidylserine oxidation/translocation. This would make them targets for macrophage recognition and phagocytosis, and thus limit their potential to invoke inflammation or give rise to neoplastic transformations. ***Key words: apoptosis/caspase-3/cumene hydroperoxide/keratinocytes/oxidative stress/phosphatidylserine/thiols. J Invest Dermatol 118:1008–1018, 2002***

In keratinocytes, the response to oxidative stimuli can be likened to the two-faced Janus. Low levels of oxidative stress are necessary prerequisites for a number of normal metabolic pathways, especially those involving signal transduction. For example, hydrogen peroxide (H_2O_2) has been invoked as a mediator in ultraviolet (UV) B-induced phosphorylation of the epidermal growth factor receptor and downstream activation of ERK1/2 and p38 in human keratinocytes (Peus *et al*, 1999). In addition, oxidative stress has been linked to controlled elevation of intracellular Ca^{2+} and activation of epidermal platelet-activating factor receptor (Travers, 1999), as well as, release of immune-modulating cytokines interleukins 1 and 6 (Tebbe *et al*, 1997). In contrast, relatively large amounts of oxidative stress are inhibitory to numerous keratinocyte functions, such as cell migration and proliferation (O'Toole *et al*, 1996). Exuberant production of reactive oxygen species can be genotoxic, and may cause direct cytotoxicity via apoptotic and/or necrotic mechanisms (Babich *et al*, 1996; Taylor *et al*, 1999).

Among different oxidative stressors, H_2O_2 and organic peroxides (including lipid hydroperoxides) are particularly important. Both H_2O_2 and lipid hydroperoxides are metabolic intermediates with important intracellular functions (O'Toole *et al*, 1996; Tebbe *et al*, 1997; Peus *et al*, 1999; Travers, 1999). In addition, organic

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Abbreviations: AAPH, azo-initiator, 2,2′-azobis(2-aminodinopropane)-dihydrochloride; Cum-OOH, cumene hydroperoxide; NHEK, normal human epidermal keratinocytes; PnA, *cis*-parinaric acid.

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hydroperoxides and alkylperoxides are commonly used as pharmaceuticals (Van Duuren *et al*, 1965; Medical Economics Co. *et al* 1997). Finally, occupational exposure of skin to different organic peroxides frequently results from their intensive use in the chemical and pharmaceutical industries (Kirk and Othmer, 1982; Adams, 1999).

Several studies reported organic peroxide-induced oxidative stress in keratinocytes (Vessey *et al*, 1992, 1995; Vessey and Lee, 1993; Babich *et al*, 1996). Whereas peroxide-induced lipid peroxidation was implicated in their toxicity as one of the essential mechanisms (Vessey *et al*, 1992; Babich *et al*, 1996), reliable data on phospholipid peroxidation in keratinocytes exposed to organic peroxides are not available. Analysis of lipid peroxidation based on the assay of thiobarbituric reactive substances failed to provide unequivocal quantitative evidence for the accumulation of oxidized lipid-derived products following exposure of keratinocytes to peroxides (Vessey *et al*, 1992). In addition, the role of low-level lipid peroxidation in signal transduction processes has received little attention in keratinocytes.

Recently, phospholipid signaling was recognized as one of the early and essential components of apoptosis (Martin *et al*, 1995; Verhoven *et al*, 1995). Transmembrane migration and externalization of phosphatidylserine, an acidic phospholipid normally confined to the inner surface of plasma membrane (Bevers *et al*, 1999), is the triggering event in the recognition of apoptotic cells by the scavenger receptors of macrophages (Fadok *et al*, 1992). This is followed by engulfment and safe digestion of apoptotic cells, thus preventing development of an inflammatory response (Fadok *et al*, 1998). Previous studies have identified site-specific oxidation of phosphatidylserine in plasma membrane as an important early step in the mechanisms leading to its externalization (Fabisiak *et al*, 1997, 1998).

As organic peroxides have the reputation of being potent skin tumor promoters and inducers of dermal hyperplasia (Kotin and Falk, 1963; Klein-Szanto and Slaga, 1982; Lai *et al*, 1996; Gimenez-Conti *et al*, 1998; Marks and Furstenberger, 2000; Zhao *et al*, 2000), their ability to trigger apoptotic cell elimination may be critical for controlling their proliferative potential. With this in mind, a detailed study of phospholipid peroxidation in normal human epidermal keratinocytes (NHEK) exposed to different concentrations of cumene hydroperoxide (Cum-OOH) was undertaken. A newly developed procedure for the analysis of site-specific phospholipid peroxidation in live cells using metabolic integration of oxidation-sensitive fluorescent fatty acid, *cis*-parinaric acid (PnA), into major classes of membrane phospholipids in NHEK (Kagan *et al*, 1998) was used to characterize Cum-OOH-induced oxidative stress in NHEK. Plasma membrane asymmetry of phosphatidylserine and phosphatidylethanolamine in NHEK exposed to Cum-OOH was also determined. Finally, these measurements were complemented with studies of different biomarkers of apoptosis (caspase-3 activation, DNA laddering and nuclear morphology). The results clearly demonstrate that Cum-OOH induced selective oxidative stress in acidic phospholipids of NHEK, namely phosphatidylserine and phosphatidylinositol, and is accompanied by phosphatidylserine externalization. Phosphatidylserine oxidation and externalization preceded the development of other characteristic biochemical steps of apoptotic machinery, such as caspase activation and DNA fragmentation. This phospholipid-based signaling pathway could therefore provide for efficient macrophage recognition and phagocytosis of NHEK following their exposure to inorganic and organic hydroperoxides.

MATERIALS AND METHODS

Reagents PnA (Z-9, E-11, E-13, Z-15-octadecatetraenoic acid) was from Molecular Probes (Eugene, OR). The purity of each lot of PnA was determined by UV spectrophotometry using the molar extinction ε_{304nm} in ethanol, 80×10^3 per M per cm. Fatty acid-free human serum albumin, polyoxyethylenesorbitan monolaurate (Tween 20), sodium molybdate, malachite green base, butylated hydroxytoluene, Hank's balanced salt solution, luminol, glutathione, Cum-OOH, and deferoxamine mesylate were purchased from Sigma (St Louis, MO). HCl, acetone, and ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA). Methanol, chloroform, hexane [high-performance liquid chromatography (HPLC) grade], 2-propanol (HPLC grade), water (HPLC grade), and sodium nitrite were purchased from Aldrich Chemical (Milwaukee, WI). Silica G plates (5×5 cm) were purchased from Whatman (Clifton, NJ). Keratinocyte growth medium (KGM)-2 was from Clonetics (San Diego, CA). Phospholipid standards were purchased from Avanti Polar Lipids (Alabaster, AL). ThioGlo-1™ was obtained from Covalent (Wobum, MA). The azo-initiator, 2,2′-azobis(2-aminodinopropane)dihydrochloride (AAPH) was from Wako Chemicals USA (Richmond, VA).

NHEK cell culture and treatment with Cum-OOH NHEK from adults were obtained from Clonetics (San Diego, CA). Cells were plated at a density of 2.5×10^3 cells per cm^2 in 75 ml tissue culture flasks (Greiner Laboratories, GmbH, Germany). NHEK were grown in KGM-2 medium at 37°C in a tissue culture incubator (5% CO_2) until 80% confluent monolayers were obtained. Repeated counts of NHEK using a hemacytometer revealed a concentration of between 1 and 2×10^6 cells per flask at the time of harvest. To induce oxidative stress, NHEK were incubated with 50 μM, 100 μM, and 200 μM Cum-OOH in the presence of deferoxamine mesylate (40 μM) in KGM basal medium without phenol red for 1 h at 37°C. After incubation, cells were washed twice with KGM-2 medium and harvested by trypsinization. NHEK homogenates were prepared by freezing at –80°C and thawing the cells.

DNA fragmentation Low molecular weight DNA fragmentation in NHEK was determined using conventional gel electrophoresis with minor modifications to the previously described methods (Fabisiak *et al*, 1997, 1998). Briefly, 10^6 cells were collected by centrifugation and washed twice with cold phosphate-buffered saline (PBS) after Cum-OOH treatment. The cell pellet was suspended in 50 μl lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM ethylenediamine tetraacetic acid, 0.5% sarkosyl) at 4°C for 15 min. Proteinase K was then added to a final concentration of 1 mg per ml and incubated overnight at 50°C. Digests were stored frozen until use, at which time 10 μg RNAse A and 200 units RNAse T_1 were added and incubated at 37°C for 1 h. The entire sample was then electrophoresed in 2% agarose gel. Gels were stained with ethidium bromide (1 μg per ml) and evaluated under UV illumination.

Nuclear morphology assay Nuclear morphology was assessed using Hoescht 33342 fluorescent staining, as previously described (Fabisiak *et al*, 1998). Control and Cum-OOH-exposed NHEK, after 1–6 h of treatment, were harvested by trypsinization, washed with PBS, fixed with 2% paraformaldehyde in PBS, and stained with Hoechst 33342 (1 μg per ml). At least 300 cells were viewed under fluorescent microscopy with excitation at 340–380 nm, and nuclear morphology was scored as either normal or apoptotic.

Electron microscopy The technique was performed using a standard protocol. Briefly, cells were grown and treated as described above. Keratinocytes were then washed with PBS, and fixed in Karnovsky Fixative (2.5% glutaraldehyde + 3% paraformaldehyde) in 1 M sodium cacodilate, pH 7.4, and postfixed with osmium tetroxide. Cells were dehydrated in graded alcohol solutions and propylene oxide and embedded in LX-112 (Ledd, Burlington, UT). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (JEOL 1220, Kyoto, Japan).

Annexin V assay for externalized phosphatidylserine Annexin V binding to cells was measured using flow cytometry essentially, as previously described (Fabisiak *et al*, 1998), with a commercially available staining kit (R&D System, Minneapolis, MN). Briefly, control NHEK and NHEK treated with Cum-OOH were washed twice in ice-cold PBS. NHEK were incubated with the annexin V–fluorescein (1 μg per ml final concentration) and propidium iodide (5 μg per ml) for 20 min at room temperature. Cells were analyzed using FACScan Becton-Dickinson (Braintree, MA) with simultaneously monitoring of green fluorescence (530 nm, 30 nm bandpass filter) for annexin V–fluorescein and red fluorescence (585 nm, 42 nm bandpass filter) associated with propidium iodide.

Assay of caspase-3 activity Caspase-3 activity in cell lysates was measured using a fluorometric assay kit obtained from Clontec (Palo Alto, CA) essentially as described in the manufacturer's instructions.

Briefly, 10^6 cells from various treatments were collected and lyzed in 50 μl ice cold lysis buffer supplied with kit. Fifty microliters of 2 × reaction buffer with 10 mM dithiothreitol were added to each lysate and kept on ice. Five microliters of 1 mM fluorescent substrate, DEVD-7-amino-4-trifluoromethyl coumarin conjugate was added to each tube and reaction started by transferring samples to individual wells of a 96-well plate (Corning, Acton, MA). Free 7-amino-4-trifluoromethyl coumarin fluorescence was determined at zero time and following 1 h incubation at 37°C using a CytoFluor 2350 (Millipore, Bedford, MA) fluorescence plate reader using excitation filter 360/40 and emission filter 530/25. Caspase-3 activity was expressed as the amount of 7-amino-4-trifluoromethyl coumarin produced within 1 h and determined from a standard curve prepared using 7-amino-4-trifluoromethyl coumarin.

Fluorescamine labeling of externalized aminophospholipids NHEK were labeled by fluorescamine as described by Fadok *et al* (1992). NHEK (10^8) were treated with fluorescamine (200 μM, final concentration) for 30 s at room temperature in 20 mM HEPES buffer containing 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM $NaHCO_3$, 5 mM glucose, pH 8.0. Afterward, 5 ml of 40 mM Tris–HCl, pH 7.4 containing 100 mM NaCl were added to remove unbound fluorescamine and cells were washed twice with the same buffer and harvested by trypsinization. Pellet of cells was resuspended in 3 ml of 40 mM Tris–HCl buffer, pH 7.4, lipids were extracted and phospholipid classes were separated by two-dimensional high performance thin-layer chromatography (HPTLC) on silica G plates (5 × 5 cm) as described earlier (Kagan *et al*, 1998).

Chemiluminescence measurements of total antioxidant reserve in NHEK A water soluble azo-initiator of peroxyl radicals, AAPH, was used to produce radicals at a constant rate (Niki, 1990). Oxidation of luminol (400 μM) by AAPH-derived peroxyl radicals in 50 mM disodium phosphate buffer (pH 7.4 at 37°C) was assayed by monitoring chemiluminescence. The reaction was started by the addition of AAPH. A delay in the chemiluminescence response, which is caused by interaction of endogenous antioxidants with AAPH-derived peroxyl radicals, was observed upon addition of NHEK homogenates (0.2 mg protein per ml). Based on the known rate of peroxyl radical generation by AAPH, the amount of peroxyl radicals scavenged by endogenous antioxidants can be determined. Luminescent analyzer 633 (Coral Biomedical, San Diego, CA) was employed for determinations.

Fluorescence assay of glutathione and protein sulfhydryls Low molecular weight thiols and protein sulfhydryl concentration in NHEK was determined using ThioGlo-1™, a maleimide reagent that produces a highly fluorescent adduct upon its reaction with SH– groups (Shvedova *et al*, 2000). Glutathione content was estimated by an immediate fluorescence response registered upon addition of ThioGlo-1 to the cell homogenate. Protein sulfhydryls were determined as an additional increase in fluorescence response after the addition of sodium dodecyl sulfate (4.0 mM) to the same cell homogenate. A standard curve was established by addition of glutathione (0.04–4.0 μM) to 100 mM phosphate buffer, pH 7.4 containing 10 μM ThioGlo-1 (dimethyl sulfoxide solution). A spectrofluorophotometer RF-5301PC (Shimadzu, Kyoto, Japan) was employed for the assay of fluorescence using excitation at 388 nm and emission at 500 nm. The data obtained were exported and treated using RF-5301PC Personal Fluorescence Software (Shimadzu).

Protein assay The protein content in NHEK was measured by Bradford method using Bio-Rad assay kit (Bio-Rad, Richmond, CA). Bovine serum albumin was used as a standard.

Determination of oxidative stress in NHEK phospholipids An oxidation-sensitive fluorescent fatty acid with four conjugated double bonds, PnA was used to label NHEK phospholipids metabolically. It was incorporated into NHEK by addition of its human serum albumin complex (5 μg PnA/10^6) to KGM-2 medium as described by Shvedova *et al* (2000). At the end of the 2 h incubation period, the NHEK were washed with KGM-2 medium with human serum albumin (0.5 mg per ml) followed by another wash with KGM-2 medium without human serum albumin to remove excess of unbound PnA. PnA-labeled NHEK were treated with Cum-OOH as described above. After that, the cells were harvested by trypsinization, lipids were extracted and resolved by HPLC as described below.

Extraction of cell lipids Total lipids were extracted from NHEK (1–2 × 10^6 cells) using the Folch procedure (Folch *et al*, 1957). The lipid extract was dried under N_2 and dissolved in 0.2 ml of 2-propanol/hexane/water (4:3:0.16, by vol.).

HPLC analysis of cell lipids Lipid extracts were separated by normal phase HPLC using a 5 μm Microsorb-MV Si column (4.6 × 250 mm) and an ammonium acetate gradient as described by Kagan *et al* (1998). The separations were performed using a Shimadzu HPLC system (model LC-600) (Kyoto, Japan) equipped with an in-line configuration of fluorescence detector (model RF-551). Fluorescence of PnA was measured at 420 nm emission after excitation at 324 nm. Data were processed and stored in digital form with Shimadzu EZChrom software. Lipid phosphorus was determined using the micromethod described by Chalvardjian and Rudnicki (1970). The amount of PnA fluorescence remaining after experimental treatments is normalized relative to that in untreated PnA-labeled control cells in order to control spontaneous basal oxidation of PnA over the incubation time.

Positional analysis of PnA incorporation into cellular phospholipids To analyze the positional distribution of PnA in membrane phospholipids, homogenates of PnA-prelabeled HL-60 cells were treated with phospholipase A_2 whose catalytic action is enhanced in the presence of mellitin. Homogenates (10^7 cells per ml) were prepared by freezing (–80°C) and thawing PnA-loaded NHEK, and treated with phospholipase A_2 from bee venom (20 U per ml) and mellitin (10 μM) in 50 mM Tris–HCl buffer pH 8.0 (containing 2 mM $CaCl_2$) at 37°C for 30 min. The reaction was terminated by the extraction of lipids by the Folch procedure (Folch *et al*, 1957). HPTLC results demonstrated that > 95% phospholipids underwent hydrolysis under the conditions used (data not shown).

Preparation of PnA-labeled liposomes Lipids were extracted from PnA-labeled NHEK as described above. The solvent was evaporated under N_2 and the film of lipids was resuspended in 20 mM HEPES buffer (pH 7.4) to achieve the lipid concentration equivalent to that used in the experiments with cells. Liposomes were performed by sonication of lipid suspension (four 15 s pulses on ice) using tip sonicator (Ultrasonic Homogenizer 4710 series, Cole-Parmer Instrument, Chicago, IL). Liposomes containing PnA-labeled phospholipids were incubated for 1 h in the presence or absence of Cum-OOH (50–200 μM) at 37°C in the dark. After incubation lipids were extracted and resolved by HPLC as described above.

HPTLC assay of phospholipid composition of NHEK Individual phospholipid classes in lipid extracts were separated by two-dimensional HPTLC on silica G plates (5 × 5 cm) as described earlier (Kagan *et al*, 1998). The phospholipid spots identified by iodine staining were scraped and transferred to tubes. Lipid phosphorus was determined by a micromethod as described by Bottcher *et al* (1961).

Statistical evaluation Data are expressed as mean ± SEM values of at least three experiments. The data were analyzed by ANOVA, and the statistical significance of differences was set at $p < 0.05$.

RESULTS

Characterization of Cum-OOH-induced oxidative stress

HPTLC of NHEK phospholipids Changes in the phospholipid composition in NHEK exposed to Cum-OOH were determined in this study. A sensitive HPTLC assay was used to resolve different classes of membrane phospholipids. It was found that phosphatidylcholine represented 40.8 ± 0.6% of the total phospholipids in the control cells, with phosphatidylethanolamine being the next most prominent phospholipid (29.3 ± 0.9%) (**Table I**). Additionally, the other phospholipids in the order of their abundance—sphingomyelin > phosphatidylinositol > phosphatidylserine > > diphosphatidylglycerol > > lysophosphatidylcholine—were detectable on the HPTLC plates. When NHEK were incubated for 1 h at 37°C in the presence of Cum-OOH (50–200 μM) no significant difference in phospholipid distribution was detected (**Table I**). The slight increase in content of lysophosphatidylcholine, a relatively minor phospholipid, in Cum-OOH-treated cells may be due to phosphatidylcholine hydrolysis, which is known to be exacerbated by oxidative stress (van Kuijk *et al*, 1985; Pacifici *et al*, 1994).

cis-PnA-Parinaric acid-based assay of oxidative stress in different classes of NHEK phospholipids The lack of HPTLC-detectable changes in the phospholipid composition of NHEK exposed to Cum-OOH might reflect effective repair of oxidized phospholipids via deacylation/reacylation pathways

Table I. Effect of Cum-OOH on phospholipid composition of NHEK[a]

Phospholipids	Content of phospholipids, % of total			
	Control	Cum-OOH		
		50 μM	100 μM	200 μM
Phosphatidylcholine	40.8 ± 0.6	40.9 ± 1.1	42.2 ± 0.5	41.6 ± 0.9
Phosphatidylethanolamine	29.3 ± 0.9	27.4 ± 1.2	25.6 ± 0.6	24.4 ± 0.6
Sphingomyelin	9.6 ± 0.4	10.4 ± 0.5	11.4 ± 0.7	10.9 ± 0.4
Phosphatidylinositol	8.6 ± 0.2	8.8 ± 0.6	8.8 ± 0.6	8.6 ± 0.4
Phosphatidylserine	7.6 ± 0.2	7.3 ± 0.2	7.0 ± 0.2	7.3 ± 0.3
Diphosphatidylglicerol	1.3 ± 0.3	1.2 ± 0.1	1.8 ± 0.1	1.9 ± 0.3
Lysophosphatidylcholine	0.4 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.1

[a]NHEK were exposed to Cum-OOH (50–200 μM) for 1 h at 37°C. At the end of incubation, cells were harvested by trypsinization, washed with PBS lipids were extracted and phospholipids resolved by HPTLC as described in *Materials and Methods*. Data are mean ± SE, n = 3.

Table II. Specific incorporation of PnA into phospholipids of NHEK[a]

Phospholipid	Incorporation of PnA (mol PnA per mol phospholipid)
Phosphatidylinositol	1:170
Phosphatidylethanolamine	1:64
Phosphatidylserine	1:152
Phosphatidylcholine	1:55

[a]NHEK were incubated in the presence of PnA (5 μg per 10^6 cells) for 2 h at 37°C. After incubation cells were washed in the presence and absence of fatty acid free human serum albumin, then lipids were extracted and resolved by HPLC.

(Van der Vliet and Bast, 1992; McLean *et al*, 1993). Therefore, the PnA-based assay that permits sensitive detection of specific oxidative stress in selected membrane phospholipids of live cells without interference from repair mechanisms (Kagan *et al*, 1998) was applied. **Table II** shows the specific incorporation of PnA into various phospholipid classes in NHEK. The molar ratio of PnA/phospholipid ranged from 1:55 for phosphatidylcholine, 1:64 for phosphatidylethanolamine, 1:152 for phosphatidylserine, and 1:170 for phosphatidylinositol. Thus, PnA labeled only a very small fraction of each of these major phospholipid classes. PnA incorporation at these levels did not appear to produce adverse effects on cells as the viability of untreated PnA-labeled cells was always greater than 95% and not different from NHEK that had not been exposed to PnA.

In mammalian cells, the *sn*-2 position in phospholipid molecules is usually occupied by polyunsaturated fatty acid residues, the preferable peroxidation substrates, whereas saturated fatty acids are located in the *sn*-1 position. This positional distribution is also essential for the maintenance of membrane integrity. Therefore, it was important to determine whether the pattern of PnA integration into molecular species of phospholipids was similar to that of other oxidizable polyunsaturated fatty acid residues. To this end, PnA-labeled phospholipids isolated from NHEK were treated with phospholipase A_2, which specifically hydrolyzes fatty acyl chains from the *sn*-2 position. HPLC analysis of these phospholipids before and after phospholipase A_2 treatment revealed that more that 99% of PnA was confined to the *sn*-2 position of all major phospholipid classes (**Table III**). In all cases less than 1% of PnA remained in phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol after phospholipase A_2 hydrolysis. Thus, PnA containing four conjugated double bonds was metabolically integrated into the *sn*-2 position in NHEK phospholipids typical of other peroxidizable polyunsaturated fatty acid residues in mammalian cell membranes. This is in line with the data in a recent paper by Drummen *et al* (1999) specifically dedicated to validation of parinarioyl phospholipids as the peroxidative indicators in model systems and cultured cells. The authors clearly demonstrated that oxidation of PnA-labeled phospholipids follows approximately the same pattern as that for arachidonoyl- or linoleoyl-containing phospholipids, the two major fatty acid residues occupying *sn-2* position. Further, as oxidative loss is determined by this procedure only in PnA-labeled phospholipids, the differences in the level of unsaturation of fatty acid residues in different phospholipid classes are eliminated as a potential source of their preferential oxidation. A serious limitation of the technique, however, is that it determines the total amounts of oxidatively modified phospholipids during the entire period of oxidative challenge independently of their ability to undergo repair via the reacylation process. As a consequence, this technique evaluates the total amount of oxidative hits accumulated in a given class of phospholipids during the entire exposure (i.e., the upper limit of oxidative phospholipid modification) and does not report the amounts of peroxidized PnA-fatty acid residues actually present in cells at any given point in time during the incubation (see also *Discussion*).

Cum-OOH (50–200 μM) was found to cause substantial concentration-dependent oxidation of all PnA-labeled phospholipids in NHEK (**Fig 1**). Phosphatidylserine and phosphatidylinositol were more sensitive to oxidation induced by Cum-OOH. At a concentration of 200 μM Cum-OOH, 80.8 ± 5.1 and 72.2 ± 8.4% of PnA-phosphatidylserine and PnA-phosphatidylinositol were oxidized, respectively. At this Cum-OOH concentration, the oxidation of PnA-phosphatidylserine was significantly greater than the oxidation of the two major NHEK phospholipids: PnA-phosphatidylcholine (54.3 ± 8.8% oxidized) and PnA-phosphatidylethanolamine (61.5 ± 7.6% oxidized) (**Fig 1**). In a separate series of experiments, liposomes prepared from PnA-labeled NHEK phospholipids were incubated with Cum-OOH to evaluate oxidation of phospholipids. Phospholipids, equivalent to that found in 10^6 cells, were incubated at 37°C for 1 h with Cum-OOH (50–200 μM). Oxidation of all classes of phospholipids in liposomes was significantly less pronounced (less than 20% PnA oxidized) than that in live NHEK (except for phosphatidylinositol) (**Fig 1**). In contrast to Cum-OOH-induced oxidation in intact living cells no selectivity for oxidation of PnA-phosphatidylserine occurred in liposomes.

Low molecular weight thiols, protein SH groups, and total antioxidant reserves in NHEK Nonprotein and protein thiol levels, as well as total antioxidant reserves in NHEK were determined to characterize further the Cum-OOH-induced oxidative stress. Addition of ThioGlo™-1 to NHEK homogenates produced an instantaneous increase in fluorescence due to the formation of the glutathione–ThioGlo-1™ adduct. The intensity of the response

Table III. Positional analysis of *cis*-PnA incorporation into normal human epidermal keratinocyte phospholipids[a]

	PnA-labeled phospholipids (ng PnA per μg total lipid Pi)	
Phospholipid	Control	Phospholipase A_2
Phosphatidylcholine	64.6 ± 3.5	0.5 ± 0.1
Phosphatidylethanolamine	40.2 ± 2.5	0.3 ± 0.1
Phosphatidylserine	4.4 ± 0.3	0.02 ± 0.01
Phosphatidylinositol	2.0 ± 0.2	0.01 ± 0.01
Lysophospholipids	ND	20.5 ± 2.2
Free *cis*-parinaric acid	1.8 ± 0.4	88.3 ± 4.8

[a]All values are mean ± SEM (n = 3). ND, not detectable. Lysophospholipids: lysophosphatidylcholine; lysophosphatidylethanolamine; lysophosphatidylserine; lysophosphatidylinositol.

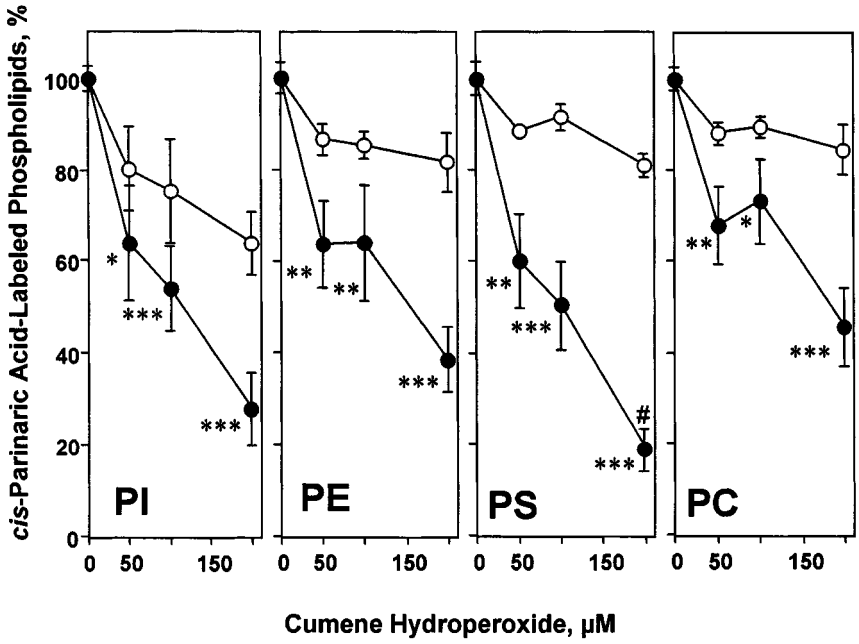


Figure 1. Effect of Cum-OOH on oxidation of PnA-labeled phospholipids in live NHEK and liposomes prepared from phospholipids of NHEK. NHEK were labeled with PnA and then incubated in phenol red-free KGM-2 basal medium in the presence or absence of Cum-OOH (50–200 μM) for 1 h at 37°C. At the end of incubation, NHEK were harvested by trypsinization, washed twice with PBS, and lipids were extracted and resolved by HPLC as described in *Materials and Methods*. Closed circles, NHEK; open circles, liposomes prepared from NHEK prelabeled with PnA. Data represent mean ± SEM obtained from five experiments. *$p < 0.01$; **$p < 0.005$, ***$p < 0.001$ *vs* control NHEK (Cum-OOH nontreated); #$p < 0.05$ *vs* phosphatidylethanolamine or phosphatidylcholine from NHEK treated with 200 μM Cum-OOH. PI, phosphatidylinositol; PE, phosphatidylethanolamine, PS, phosphatidylserine, PC, phosphatidylcholine.

remained constant until sodium dodecyl sulfate was added to the incubation system at which point a slow increase of fluorescence was observed that reached a plateau within 45–60 min. Sodium dodecyl sulfate denatures the various proteins allowing access of ThioGlo^{TM}-1 to protein SH groups, hence this latter fluorescence response reflects the amount of protein SH groups (Kagan *et al*, 1999; Shvedova *et al*, 2000). As shown in **Table IV** the exposure to Cum-OOH produced a significant concentration-dependent decrease in NHEK glutathione concentration of 18.1%, 21.0%, and 25.5% from the initial level after 1 h incubation in the presence of 50 μM, 100 μM, and 200 μM Cum-OOH, respectively. Similarly, the content of protein SH groups decreased in a concentration-dependent manner in NHEK incubated with Cum-OOH (**Table IV**).

In order to assess the potential contribution of glutathione recycling, partly to replenish oxidized glutathione experiments were performed with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), an inhibitor of glutathione reductase. Glutathione content in BCNU-pretreated cells was measured before and after exposure to 200 μM Cum-OOH. BCNU pretreatment alone produced approximately a 30% consumption of glutathione from 150.2 ± 12.0 to 115.1 ± 10.0. No significant change in protein SH groups was observed (221.5 ± 20.0 *vs* 210.8 ± 15.0). When BCNU-treated cells were exposed to Cum-OOH glutathione levels fell to 85.1 ± 7.0, a level that represents the additive sum of the losses observed with each agent individually. Therefore, BCNU pretreatment did not significantly change the extent of glutathione loss by Cum-OOH. Moreover, the loss of protein SH groups in NHEK exposed to a combination of BCNU and Cum-OOH was the same with Cum-OOH alone suggesting that the loss of protein SH groups was solely the result of Cum-OOH.

Surprisingly, the pronounced oxidation of glutathione, protein SH groups, and phospholipids was accompanied with only slight consumption of total antioxidant reserves as evidenced by chemiluminescence-based measurements of NHEK antioxidants capable of scavenging AAPH-derived peroxyl radicals. Calculations based on the rate constant for AAPH decomposition (Niki, 1990) and experimentally determined duration of the lag periods in the chemiluminescence response due to luminol oxidation, showed that antioxidants in nontreated NHEK were able to scavenge 207.9 ± 12.6 nmol peroxyl radicals per mg protein. In exposed NHEK, total antioxidant reserves were decreased such that 186.6 ± 11.6, 183.2 ± 8.5, and 181.9 ± 6.2 nmol peroxyl radicals per mg protein could be scavenged after incubation with 50 μM, 100 μM, and 200 μM Cum-OOH, respectively (**Table IV**).

Characterization of Cum-OOH-induced apoptosis in NHEK

Externalization of phosphatidylserine Cells undergoing apoptosis in response to a variety of stimuli, including oxidative stress, rapidly externalize phosphatidylserine to the outer membrane surface. Therefore, externalization of phosphatidylserine in NHEK exposed

Table IV. Effect of Cum-OOH on antioxidant reserves and content of glutathione and protein thiols in NHEK[a]

Cum-OOH (μM)	Antioxidant reserves[b] (nmol per mg protein)	Glutathione (nmol per mg protein)	Protein thiols (nmol per mg protein)
0.0	207.9 ± 12.6	150.2 ± 12.0	221.5 ± 20.0
50.0	186.6 ± 11.6	123.0 ± 10.0*	169.4 ± 11.0*
100.0	183.2 ± 8.5	118.7 ± 15.0*	143.8 ± 18.0*
200.0	181.9 ± 6.2	111.9 ± 14.0*	129.3 ± 14.0*

[a]NHEK were incubated in phenol red free KGM-2 basal medium in the presence or in the absence of Cum-OOH for 1 h at 37°C. After incubation cells were harvested by trypsinization, pelleted by centrifugation, and washed twice with PBS. Cell homogenates were prepared and used for determination of antioxidant reserves by chemiluminescence of luminol in the presence of AAPH or for measurement of thiols by ThioGlo™-1. Data are mean ± SEM,
*$p < 0.05$ *vs* control cells (no Cum-OOH added), n = 6.
[b]Antioxidant reserves expressed as AAPH-derived peroxyl radicals scavenged by NHEK homogenate (nmol per mg protein).

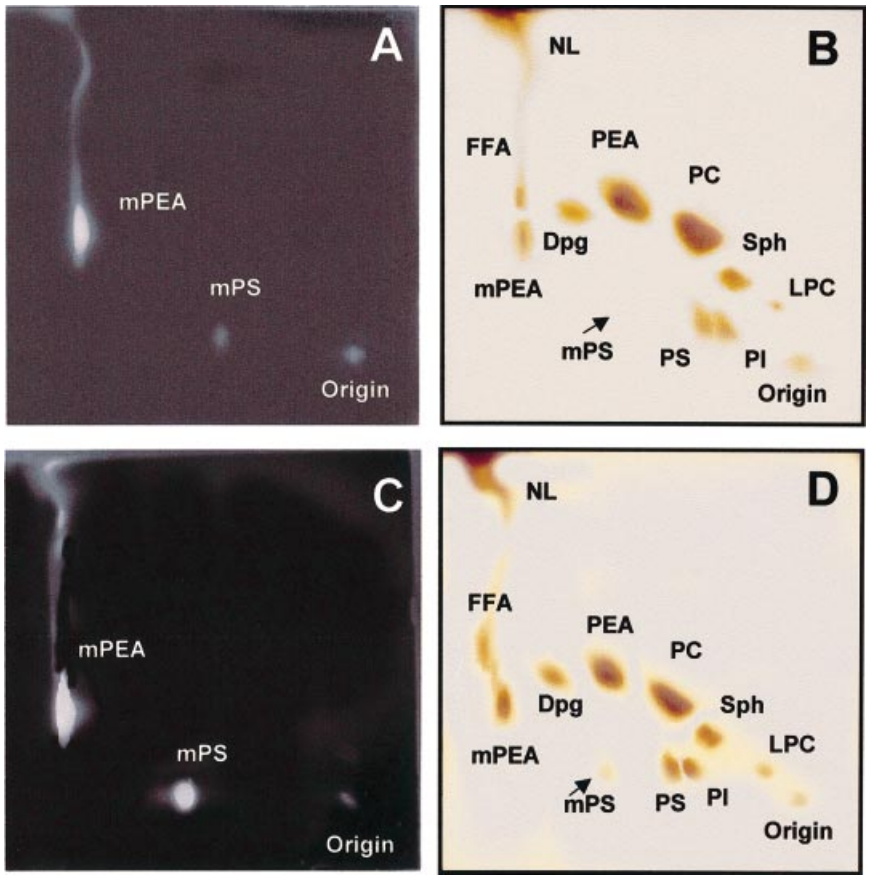


Figure 2. HPTLC analysis of total and fluorescamine-modified phospholipids from control and Cum-OOH-exposed NHEK. Total lipid extracts from control (*A*, *B*) and Cum-OOH-treated NHEK (*C*, *D*) were subjected to two-dimensional HPTLC. Identities of lipids are: NL, neutral lipids; FFA, free fatty acids; PEA, phosphatidylethanolamine; phosphatidylcholine, phosphatidylcholine; Sph, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; Dpg, diphosphatidylglycerol; mPEA, fluorescamine-modified phosphatidylethanolamine; mPS, fluorescamine-modified phosphatidylserine. The chromatograms were visualized using a Bio-Rad Fluor-S Multiimager equipped with a UV vis light source. (*A*, *C*) Fluorescence of fluorescamine-modified lipids. (*B*, *D*) Total lipids after exposure to iodine vapors.

to Cum-OOH was measured. Two different approaches were used to characterize phosphatidylserine externalization quantitatively. To estimate the amount of phosphatidylserine accessible to chemical modification on the cell surface NHEK were treated with fluorescamine, a cell impermeable amino-reagent, after which the lipids were extracted and resolved by HPTLC. The distinct spots of modified phosphatidylethanolamine and modified phosphatidylserine, as well as, unmodified phosphatidylserine and phosphatidylethanolamine were scraped. Determination of Pi content in these individual spots was performed. The amounts of modified phosphatidylethanolamine and modified phosphatidylserine were quantified relative to the total phosphatidylethanolamine and phosphatidylserine, respectively. Two representative chromatograms from control and Cum-OOH-treated cells are shown in **Fig 2**. Panels A and B show the HPTLC plates obtained from control cells visualized with exposure to UV light for the detection of fluorescamine-modified phospholipids and by exposure to iodine vapor for total phospholipids, respectively. Note that significant fluorescamine-modified phosphatidylethanolamine was detected but only a trace of modified phosphatidylserine could be found in control cells. In contrast, a significant specific accumulation of fluorescent modified phosphatidylserine was observed after exposure of cells to Cum-OOH (**Fig 2*C***). A comparison of the total phospholipid profiles between control (**Fig 2*B***) and Cum-OOH-exposed (**Fig 2*D***) cells indicates nearly identical distribution and amount of total phospholipids between these conditions. The amount of modified phosphatidylserine and modified phosphatidylethanolamine were quantified and as shown in **Fig 3**, only trace amounts of total phosphatidylserine (0.78 ± 0.06%) were accessible to fluorescamine on the cell surface of control (untreated) cells. Cum-OOH (50 μM and 200 μM) produced an 8- and 10-fold increase in the amount of phosphatidylserine available to fluorescamine after 1 h incubation, respectively. The amount of phosphatidylethanolamine on the

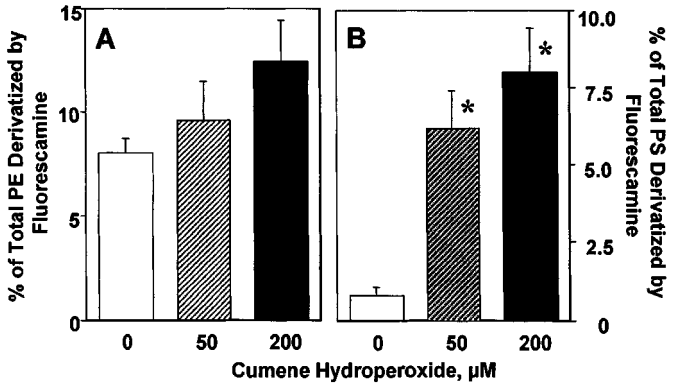


Figure 3. Effect of Cum-OOH on externalization of phosphatidylserine in NHEK. NHEK were incubated in phenol red-free KGM-2 basal medium in the presence or absence of Cum-OOH (50 or 200 μM) for 1 h at 37°C. After the end of incubation, NHEK were treated with fluorescamine for 30 s, washed, harvested by trypsinization, and lipid were extracted and resolved by HPTLC as described in *Materials and Methods*. Panel A shows the amount of derivatized phosphatidylethanolamine (PE) and panel B shows the amount of derivatized phosphatidylserine (PS). Data are expressed as a percent of the total (unmodified + modified) pool of that particular phospholipid. Data represent mean ± SEM obtained from three experiments. *$p < 0.05$ *vs* control (NHEK nontreated with Cum-OOH).

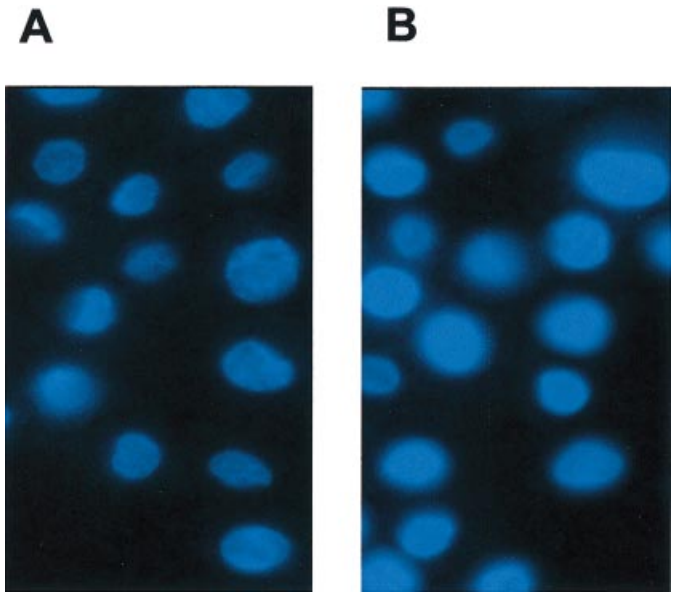


Figure 4. Nuclear morphology of normal and Cum-OOH-exposed NHEK. NHEK were incubated in phenol red-free KGM-2 basal medium in the absence (*A*) or in the presence (*B*) of Cum-OOH (200 μM) for 1 h at 37°C. Following incubation Cum-OOH was washed out and NHEK were incubated in fresh KGM-2 basal medium for an additional 5 h at 37°C. NHEK were then harvested by trypsinization, washed with PBS, fixed with 2% paraformaldehyde in PBS, and stained with Hoechst 33342. Photomicrograph is representative of three experiments.

outer surface of cell membrane was not significantly different between untreated and treated cells and was estimated as 7.99 ± 0.83% for control cells and 9.56 ± 2.0% and 12.4 ± 2.1% for cells treated with 50 μM and 200 μM Cum-OOH, respectively. These results are in line with the data in the literature indicating that phosphatidylserine is uniquely confined to the inner leaflet of plasma membrane in normal cells and undergoes early selective externalization during apoptosis (Fadok *et al*, 1992, 1998).

Using the annexin V-binding assay that characterizes the fraction of cells expressing externalized phosphatidylserine it was found that exposure of NHEK to Cum-OOH (200 μM, 1 h) resulted in a more than 2-fold increase of the amount of annexin V-positive/propidium iodide-negative cells (data not shown). This supports our finding that Cum-OOH induces phosphatidylserine externalization in NHEK.

Viability and apoptosis in NHEK Further investigations were conducted to determine whether Cum-OOH-induced oxidative stress and phosphatidylserine oxidation/externalization were associated with the appearance of other typical biochemical features of apoptosis such as caspase-3 activation, DNA fragmentation, and morphologic changes in NHEK. Chromatin condensation and fragmentation is an established biomarker of late apoptosis. We first attempted to visualize Cum-OOH changes in NHEK nuclear morphology. It was found that NHEK had not acquired a nuclear phenotype characterized by chromatin condensation and fragmentation into discrete bodies typical of apoptosis after 1 h exposure to 50–200 μM Cum-OOH (**Fig 4**). In line with this, transmission electron microscopic examination of NHEK incubated with 200 μM (400 nmol per 10^6 cells) Cum-OOH for 2 h at 37°C did not reveal any ultrastructural changes in cell morphology (**Fig 5**). The nucleus and cytoplasmic organelles of treated NHEK did not show any changes compared with the controls. Mitochondria, tonofilaments, and other cytoplasmic organelles were visible and also appeared normal.

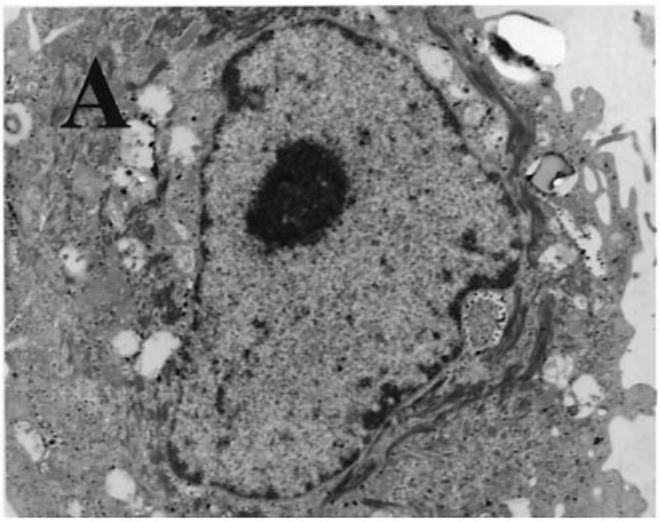
Analysis of DNA fragmentation, another hallmark of late apoptosis, was also conducted. Similar to the results with nuclear morphology, no internucleosomal DNA cleavage was detected within 5 h after a 1 h treatment with Cum-OOH in the range of concentrations from 50 to 200 μM (data not shown). Thus, the oxidation and externalization of phosphatidylserine was not accompanied by simultaneous other established end-points of apoptosis.

It is well known that one of the important biochemical steps that precedes and is required for the initiation of a number of apoptotic end-points is caspase activation. Therefore, the temporal relationship between phosphatidylserine oxidation/externalization and caspase proteolytic activity was assessed. A significant increase in caspase-3 activity could only be detected 5 h after exposure to Cum-OOH (200 μM, for 1 h) (**Fig 6**). Neither lower concentrations of Cum-OOH measured 5 h after exposure nor 200 μM Cum-OOH at earlier time points caused significant effects on caspase-3 activity.

Time-dependent changes in NHEK viability were assayed using the Trypan Blue exclusion test. Cells were exposed to various concentrations of Cum-OOH for 1 h and then media were replaced with fresh media without Cum-OOH and incubated for various times. **Figure 7** shows that a significant loss of viability was observed only 5 h after exposure to Cum-OOH (200 μM, 1 h). Viability of NHEK exposed to 50 μM Cum-OOH at 5 h or to 50–200 μM at earlier time points (3 h) was not different from that in untreated control cells.

DISCUSSION

Occupational exposure of skin cells to organic peroxides has been associated with an increased risk of cancer due to their ability to act as tumor promoters and inducers of dermal hyperplasia likely arising from their ability to cause oxidative stress (Klein-Szanto and Slaga, 1982; Kensler *et al*, 1995; Zhao *et al*, 2000). In addition, organic peroxides are commonly used as a standard experimental model of clinically relevant oxidative stress-based skin diseases. In fact, a recent study demonstrated that repeated administration of benzoyl peroxide produces skin changes in the hairless mouse that qualitatively resemble those produced by UVB and suggest that common mechanisms may be involved (Ibbotson *et al*, 1999).



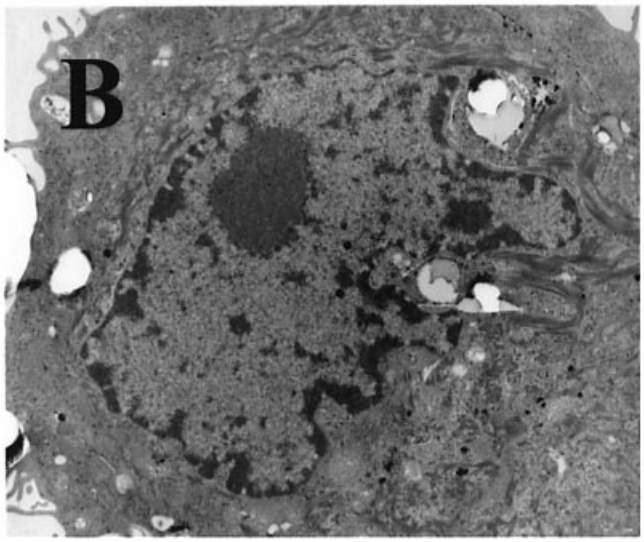


Figure 5. Electron microscopy of NHEK treated with Cum-OOH. Typical electron micrographs of control NHEK (*A*) and NHEK treated with 200 μM Cum-OOH for 2 h (*B*) are shown. No morphologic distinctions in the nucleus or cytoplasm were evident between the Cum-OOH exposed and control NHEK. View of normal ultrastructural appearance of nucleus and nucleolus. Original magnification × 6750. Conditions: NHEK (5×10^6) were treated with. Cum-OOH-treated and control cells were fixed immediately after exposures to 200 μM Cum-OOH or vehicle, respectively, at 37°C in CO_2 incubator in phenol red-free KGM-2 basal medium for 2 h.

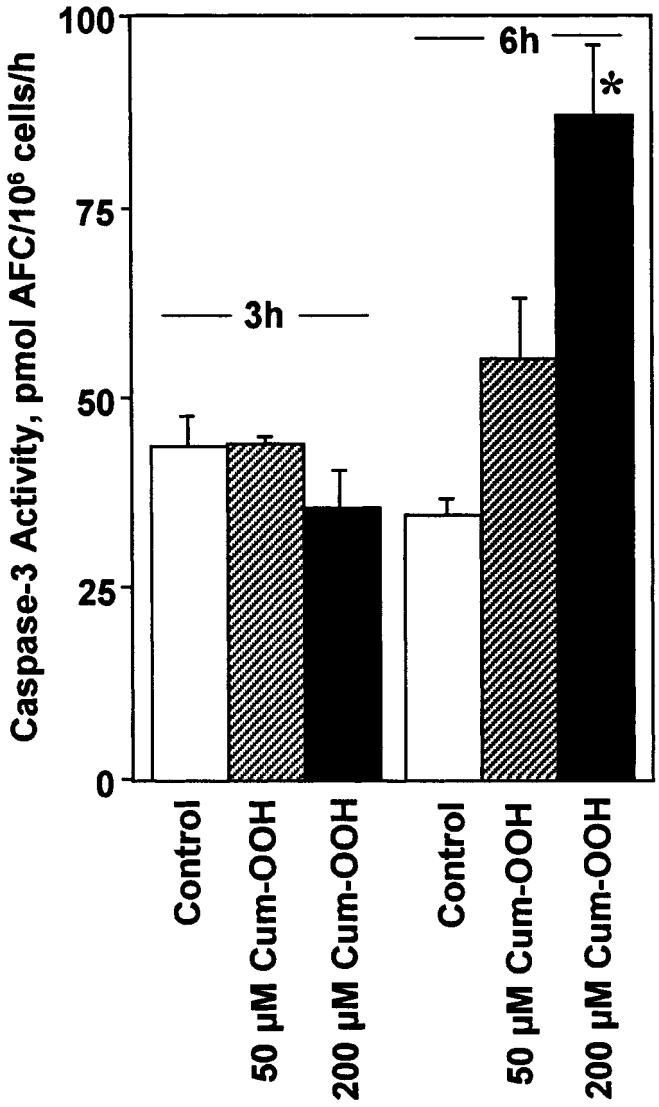


Figure 6. Effect of Cum-OOH on caspase-3 activity in NHEK. NHEK were incubated in phenol red-free KGM-2 basal medium in the presence or absence of Cum-OOH (50 or 200 μM) for 1 h at 37°C. After that, Cum-OOH was washed out and NHEK were incubated in fresh KGM-2 basal medium for 5 h at 37°C. NHEK were then harvested by trypsinization and activity of caspase-3 was determined as described in *Materials and Methods*. *$p < 0.05$ *vs* control (NHEK nontreated with Cum-OOH). Data represent mean ± SEM obtained from three experiments.

In support of this, studies from several laboratories demonstrated that exposure of keratinocytes to organic hydroperoxides or alkylperoxides results in the production of electron paramagnetic resonance-detectable free radical intermediates (such as methyl radicals) (Iannone *et al*, 1993), depletion of major intracellular antioxidants (such as glutathione) (Vessey *et al*, 1992, 1995; Vessey and Lee, 1993; Babich *et al*, 1996), and oxidative modification of DNA bases (e.g., accumulation of 8-OHdG) (King *et al*, 1996). Surprisingly, however, a number of studies have failed to reveal any detectable lipid peroxidation in keratinocytes even after exposure to high millimolar concentrations of peroxides (Vessey *et al*, 1992; Babich *et al*, 1996). Most of this previous work with keratinocytes has utilized a lipid peroxidation assay based upon 2-thiobarbituric acid to detect 2-thiobarbituric acid-reactive substances. This methodology has been extensively criticized for its inability to characterize quantitatively lipid peroxidation both *in vivo* and *in vitro* (Kagan, 1988; Moore and Roberts, 1998). Given that unsaturated membrane phospholipids are the most preferable targets for oxidative stress (Kagan, 1988) the reported absence of peroxide-induced lipid peroxidation demands further verification by detailed studies using alternative methodologies.

A novel sensitive and specific assay for peroxidation of different classes of membrane phospholipids in live cells was recently developed (Ritov *et al*, 1996; Kagan *et al*, 1998). This assay is based on metabolic integration of *cis*-PnA, a natural 18-carbon fatty acid

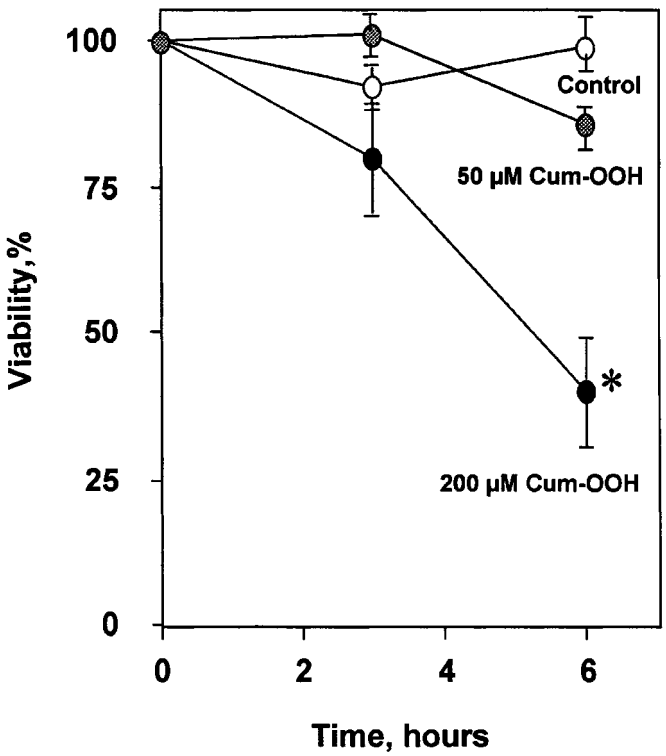


Figure 7. Viability of NHEK following exposure to Cum-OOH. Cells were exposed to Cum-OOH for 1 h and then media changed to fresh media without Cum-OOH. Viability was assessed by Trypan Blue at indicated times after exposure. Data represent mean ± SEM obtained from four experiments. *$p < 0.05$ *vs* control.

with four conjugated double bonds, into membrane phospholipids of cells. This conjugated double bond system confers highly fluorescent properties to PnA and renders it highly susceptible to peroxidation. Oxidation of PnA results in disruption of the conjugated double bond system that cannot be re-synthesized in mammalian cells (Kuypers *et al*, 1987; van den Berg *et al*, 1991). Therefore, upon peroxidation, fluorescence is irreversibly lost from specific classes of phospholipids. Different versions of PnA-based fluorescence assays of lipid peroxidation have been successfully used in studies of oxidative stress in keratinocytes (Carini *et al*, 2000; Peus *et al*, 2000) and a number of other cell lines (Miro *et al*, 1999; Ross *et al*, 1999; Tiku *et al*, 2000). These workers, however, did not utilize metabolic integration of PnA into different classes of phospholipids and, therefore, did not address the issue of phospholipid-specific peroxidation.

In this study, therefore, phospholipid peroxidation in keratinocytes exposed to moderate concentrations of organic peroxides (Cum-OOH) was determined using metabolic integration of PnA into membrane phospholipids with their subsequent HPLC separation and fluorescence detection. It was found that keratinocytes readily integrated PnA into major classes of membrane phospholipids such that molar ratios of PnA-labeled phospholipids in different classes were 1.8 mol%, 1.5 mol%, 0.7 mol%, and 0.5 mol% for phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol, respectively. Thus, the fractions of PnA-labeled phospholipids in each of the classes were not large enough to disturb the biophysical characteristics of membranes but were sufficient for detecting their oxidation. In fact, exposure to the highest concentration of Cum-OOH did not completely consume phospholipids in any of the PnA-labeled classes suggesting that by the end of the incubation there was still enough oxidizable substrate and the amount of the PnA-labeled phospholipids was not a limiting factor. In contrast to the data in the literature (Vessey *et al*, 1992; Babich *et al*, 1996), Cum-OOH was remarkably effective in inducing peroxidation of PnA-labeled membrane phospholipids in NHEK.

Peroxidized phospholipids are known to be readily attacked by phospholipases A_2 yielding lysophospholipids and fatty acid oxidation products (e.g., fatty acid hydroperoxides) (Rashba-Step *et al*, 1997). The former are effectively reacylated, i.e., undergo remodeling, whereas the latter may further decompose via β-scission giving rise to esterified short-chain fatty acid fragments likely containing a carbonyl function or enzymatically converted to lipid alcohols by glutathione-based peroxidases (Kagan, 1988). As shown in **Table I**, exposure to Cum-OOH did not result in any significant accumulation of lysophospholipids indicating that effective reacylation was likely involved in the repair of oxidatively modified phospholipids. This suggests that the level of phospholipid peroxidation detected was compatible with the continued functioning of physiologic repair mechanisms. In fact, no significant changes in NHEK viability were found until 5 h after incubation and even then only with the highest Cum-OOH concentration used.

Whereas these results indicate that remodeling of oxidatively modified parinarioylated phospholipids, in contrast to endogenous phospholipids, does not interfere with the quantitative assay of Cum-OOH-induced oxidative stress in different classes of NHEK phospholipids, they also suggest that one has to be cautious with the interpretation of the data. This is because the cumulative nature of the assay reports the total amount of oxidatively modified PnA-phospholipids at least a part of which might have been remodeled in the course of oxidative stress. It should be also noted that our results revealed preferential Cum-OOH-induced oxidation of PnA-phosphatidylserine a relatively small fraction of total endogenous NHEK phosphatidylserine as compared with phosphatidylserine containing natural polyunsaturated fatty acid residues. This raises a concern that there may be a biased interpretation of the results because detectable oxidation was found only in PnA-phospholipids. Whereas there is no direct data to address this issue several lines of evidence are supportive of our explanation. First, previous work has demonstrated that oxidation of PnA-labeled phospholipids in cells follows approximately the same pattern as that for arachidonoyl- or linoleoyl-containing phospholipids (Ritov *et al*, 1996; McGuire *et al*, 1997; Drummen *et al*, 1999). Second, Cum-OOH induced nonrandom peroxidation of NHEK phospholipids. Selectively higher sensitivity of NHEK PnA-labeled phosphatidylserine (and phosphatidylinositol) as compared with phosphatidylcholine and phosphatidylethanolamine supports the involvement of specific redox catalytic mechanisms as PnA itself would be equally sensitive to oxidation regardless of the polar head group contained on that phospholipid. Finally, in separate experiments, it was found that PnA labeling of phospholipids did not affect Cum-OOH-induced externalization of phosphatidylserine in NHEK as determined by fluorescamine-based assay (data not shown). Thus phosphatidylserine-dependent signaling cascade of the apoptotic program was effective both in the presence and absence of PnA. It is tempting to speculate that the reactivity of PnA-phosphatidylserine adequately reflects behavior of phosphatidylserine in the natural phospholipid population in NHEK, although the integral cumulative character of PnA-based assay should be kept in mind.

The presence of efficient glutathione recycling mechanisms had only limited effects on Cum-OOH-induced oxidation of glutathione and protein SH groups. This became apparent from our experiments with BCNU plus Cum-OOH in which BCNU acted additively, but not synergistically, with Cum-OOH. This is in line with the potential involvement of glutathione in the repair of Cum-OOH-induced oxidation of lipids via a glutathione peroxidase catalyzed reduction of lipid hydroperoxides to their respective more stable hydroxy homologs at the expense of glutathione oxidation. One can speculate that loss of glutathione associated with the repair of oxidized lipids was not as readily recyclable through BCNU-sensitive pathways. Regardless, these data imply

that, indeed, phospholipids are preferable substrates for peroxidative stress in NHEK.

An HPTLC technique was utilized in this study that permits the quantitative analysis of relatively small amounts (25–50 μg) of lipids isolated from cells. The relatively high sensitivity of the method and its accuracy within 3–5% is compatible with the detection of 5–7% changes in phospholipid composition. In fact, by using the same HPTLC procedure it has been able to detect reliably externalization of ≈6% phosphatidylserine of its total content in cells, i.e., 0.4% of total phospholipids in NHEK. Thus, our quantitative HPTLC analysis of cell phospholipids was used to reveal changes in relative distribution of different classes of phospholipid after oxidative challenge. Within the accuracy of our assay, no significant changes in patterns of phospholipid distribution were found. Thus it was concluded that no massive changes in phospholipid composition of phospholipids were induced by Cum-OOH within the exposure time intervals and concentrations used. Based on this, a more sensitive method was utilized for the detection of peroxidative modification of phospholipids based on their metabolic labeling with oxidation-sensitive PnA. In this case, it was possible to detect significant peroxidation that in quantitative terms has occurred selectively in different classes of phospholipids. Therefore, these results were interpreted as suggestive of potent repair and remodeling of oxidatively modified phospholipids, such that no changes in relative abundance of phospholipids was noticeable in live cells. The repair process includes removal of oxidized fatty acid residues from phospholipids by phospholipases A_2 and subsequent enzymatic reacylation of lysophospholipids formed (Rashba-Step *et al*, 1997; Van der Vliet and Bast, 1992; McLean *et al*, 1993).

Importantly, it was established that different phospholipids are oxidized nonrandomly after exposure of NHEK to Cum-OOH. The most profound oxidation was detected in phosphatidylserine and phosphatidylinositol—two anionic phospholipids. Phosphatidylethanolamine and phosphatidylcholine, the two most abundant phospholipids in membranes contained, proportionally, much more PnA-labeled species and were oxidized to a significantly less extent. Thus, the extent of oxidation was not determined by the prevalence of various phospholipids in NHEK. Moreover, in liposomes prepared from NHEK phospholipids, the pattern of Cum-OOH-induced oxidation followed essentially a random pattern indicating that site-specific phosphatidylserine oxidation was characteristic for hydroperoxide effects in intact living cells.

Previous work indicated that selective phosphatidylserine oxidation was a characteristic early event in oxidant-induced apoptosis and that this preceded phosphatidylserine externalization in several models of oxidant-induced apoptosis (Kagan *et al*, 2000; Tyurina *et al*, 2000). Selective oxidation of phosphatidylserine during apoptosis was inhibited by overexpression of *bcl*-2 (Fabisiak *et al*, 1997; Tyurina *et al*, 1997) and was not inhibited by the vitamin E analog, 6-hydroxy-2,2,5,7,8-pentamethylchromane (Fabisiak *et al*, 1998). Moreover, it appears that macrophage recognition of apoptotic cells is dependent, in part, on phagocyte expression of oxidized/modified low-density lipoprotein receptors (Fadok *et al*, 1998) and at least one enzyme involved in the maintenance of plasma membrane phospholipid asymmetry is inhibited by redox reactions within the lipid bilayer (Herrmann and Devaux, 1990; Fabisiak *et al*, 2000). Thus, it can be speculated that phosphatidylserine oxidation is a specific part of the apoptotic pathway following oxidative stress and participates in the modulation of phosphatidylserine translocation and its eventual interaction with macrophage cell surface receptors.

Similarly, in this study, it was found that Cum-OOH induced externalization of phosphatidylserine on the surface of NHEK, as evidenced by its increased accessibility to fluorescamine and annexin V binding. This effect was phosphatidylserine specific, as no significantly increased phosphatidylethanolamine externalization was observed in Cum-OOH-exposed NHEK. By focusing on these early cellular responses, other typical markers of apoptosis were not detected in association with phosphatidylserine oxidation and translocation. It has been noted, however, that phosphatidylserine oxidation and translocation occur very early in the genesis of apoptosis and, hence, often precede other markers of apoptosis (Fadok *et al*, 1992; Martin *et al*, 1995, Reno *et al*, 1998; Kagan *et al*, 2000; Tyurina *et al*, 2000). In addition, phosphatidylserine-specific events can often be dissociated from other aspects of apoptosis, an observation that suggests the ability of these events to occur independent of caspase activation (Drenou *et al*, 1999; Carmody and Cotter, 2000). Even if phosphatidylserine oxidation and translocation, as observed here in NHEK, occurs independent of apoptosis, its importance is underscored by the likelihood that oxidatively damaged keratinocytes would most likely be recognized and eliminated by phagocytosis, thus, limiting the potential of neoplastic transformation. Therefore, phosphatidylserine oxidation/translocation may represent a protective mechanism within the skin following oxidant challenge.

In summary, these results clearly demonstrate that Cum-OOH induces significant oxidative stress in NHEK phospholipids characterized by early and profound oxidation of phosphatidylserine associated with phosphatidylserine externalization. As externalized phosphatidylserine is a signal for recognition of apoptotic cells by macrophage scavenger receptors it can be speculated that phosphatidylserine oxidation may be translatable into elimination of oxidatively damaged NHEK. Further studies are required to establish quantitative interrelationships between the amounts of oxidized and externalized phosphatidylserine necessary for the engulfment and digestion of NHEK by professional macrophage cells, a process that may attenuate the procarcinogenic potential of organic peroxides.

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